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Immunochemical Determination of 2,4,6-Trichloroanisole as the Responsible Agent for the Musty Odor in Foods. 2. Immunoassay Evaluation

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Immunoassays for 2,4,6-trichloroanisol (TCA) have been evaluated. The assays were developed after raising antibodies against three different immunizing haptens (*1*). Lack of reproducibility has been one of the main problems of these assays. Precision was worse on these assays, reaching lower limits of detection. The high lipophilicity of TCA and its, consequently, low water solubility have been found to be the major cause of this problem. A reliable microplate-based enzyme-linked immunosorbent assay (ELISA) has been set after consideration of the TCA physicochemical features and evaluation of important parameters affecting immunoassay performance. The immunoassay uses As78 (developed against hapten **B**-KLH) and **C9**-OVA as the coating antigen. The selectivity is high although the brominated analogue 2,4,6-TBA is also recognized. In buffered media containing 7% ethanol, the resulting assay shows a good accuracy with an IC_{50} value of 0.53 μg L^{-1} and a limit of detection of 0.044 μg L^{-1}. Red and white wine samples caused important interferences in the immunoassay demonstrating the necessity of a cleanup procedure prior to the ELISA.

KEYWORDS: Trichloroanisole; musty odor; wine; cork; immunoassay; lipophilicity

INTRODUCTION

2,4,6-Trichloroanisole (2,4,6-TCA) has been identified as the cause of the musty odor problems in beverage and food products. Although other compounds may also contribute to such undesirable odors, it has been found that when 2,4,6-TCA is the agent responsible, this musty aroma can be detected at very low concentration levels (10−50 ppt). This specific problem, called "cork taint" in the wine industry, has been traditionally associated with the use of cork stoppers (*2*−*4*). It has been assumed that the nature of the cork stoppers and the traditional manufacturing process favor the growth of certain molds responsible for the formation of TCA through methylation of the trichlorophenol (TCP), frequently used as a preservative. However, the presence of these molds in the wine cellars has also been reported, suggesting that TCA contamination may also occur during wine production (*5*, *6*).

Cork and wine industries need suitable and efficient monitoring methods in order to prevent cork taint in the marketed final products. Current analytical methods are too laborious considering the detectability requirements and the high sample load of this industrial sector. A medium size company manufacturing cork stoppers may produce more than 5×10^5 stoppers by day, which means that nearly 700 cork stoppers should be analyzed (ISO-2859-I) each day. Immunoassay techniques are analytical tests based on the specific interaction between the antibody and the antigen. Because of their high sensitivity and selectivity, immunoassays have been successfully used for both qualitative and quantitative analysis of several substances at trace levels (*7*−*10*). Additionally, immunoassays are characterized by their low cost, rapidity, simplicity, and possibility to process high sample loads (*11*−*13*). In our preceding paper (*1*), we reported the production of polyclonal antibodies against 2,4,6-TCA considering the influence of the immunizing hapten chemical structure on the resulting immunoassay. In this paper, we present the evaluation of these immunoassays, paying particular attention to the effect of the TCA physicochemical features on the reliability of the immunoassay.

EXPERIMENTAL SECTION

General Methods and Instruments. The pH and the conductivity of all buffers and solutions were measured with a pH 540 GLP pH meter and a LF 340 conductimeter, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, DK). Washing steps were carried out using a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read on a SpectramaxPlus microplate reader (Molecular Devices, Sunnyvale, CA). The competitive curves were analyzed with a four parameter logistic equation using the software SoftmaxPro v2.6 (Molecular Devices) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

Chemicals and Immunochemicals. Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Standards for cross-reactivity studies were purchased from Aldrich Chemical Co. (Mil-

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waukee, WI). Stock solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 mM. The preparation of the antisera As74−As76 (**A**-KLH), As77−As79 (**B**-KLH), and As88−A90 (**C**-KLH) and of the immunoconjugates (**C14**-CONA, **C9**-OVA, and **C**-OVA) has already been described in our previous paper (*1*). The coating antigens were stored freeze-dried at −40 °C. Working aliquots with the necessary amounts to prepare two microtiter plates were prepared in Eppendorf tubes and stored freeze-dried at 4 °C.

Buffers. Unless otherwise indicated, phosphate-buffered saline (PBS) is 0.01 M phosphate buffer and 0.8% saline solution and the pH is 7.5. PBST is PBS with 0.05% Tween 20. PBST−EtOH is PBS with 7% ethanol and 0.005% Tween 20, and the pH was adjusted to 8.5. Borate buffer is 0.2 M boric acid−sodium borate, pH 8.7. The coating buffer is 0.05 M carbonate−bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% tetramethylbenzidine and 0.004% H_2O_2 in citrate buffer.

Wine Samples. White and red representative wines were used to assess the effect of the matrix in the optimized immunoassay. Both were Spanish wines (Viña Mar, cosecha 2000) that had been analyzed by solid phase microextraction/gas chromatography/mass spectrometry (SPME/GC/MS) to ensure the absence of TCA (*14*).

Competitive Indirect ELISA. *General Protocol.* Unless otherwise indicated, all of the experiments were performed according to this protocol. The microtiter plates were coated overnight at 4 °C with the coating antigen appropriately diluted in coating buffer (100 μL/well). The day after, the plates were washed four times with PBST and 12 serial dilutions of 2,4,6-TCA (10 000 nM to 1 pM, in PBST, 50 μL/well) were added to the coated plates followed by the antisera appropriately diluted in PBST (50 μL/well). The mixture was incubated for 30 min at room temperature (RT), and the plates were washed as described before. A solution of goat anti-rabbit IgG coupled to horseradish peroxidase (antiIgG-HRP, 1/6000 in PBST) was added to the wells (100 μL/well) and incubated for 30 min at RT. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at RT with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm. The standard curve was fitted to a four parameter logistic equation according to the following formula: $Y = [(A - B)/1 - (x/C)^D] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve.

As78/C9-OVA ELISA Evaluation. Different experimental parameters, length of the competitive step, blocking agents, effect of the pH, effect of the ionic strength, and presence of ethanol were studied sequentially in this order using the above-described protocol. However, each time an evaluated parameter was changed, this new value was used for the evaluation of the next condition.

As78/C9-OVA. *Optimized ELISA.* Microtiter plates were coated with **C9**-OVA in coating buffer (0.315 μg mL^{-1}, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The following day, the plates were washed with PBST (four times, 300 μL/well) and a solution of 1% of polyvinylpyrrolidone (PVP) in PBST was added to the wells (100 μL/well) and incubated for 30 min at RT. After the plates were washed as described before, 2,4,6-TCA standards (1000 μM to 6.4 nM, prepared in DMSO) were diluted 200 times with PBST−EtOH (0.01 M PBS, pH 8.5, with 0.005% Tween 20) containing the As78 (1/2000 in PBST−EtOH) and added to the microtiter plates (100 μL/well). After 20 min of incubation time at RT, the plates were washed again as before and a solution of anti IgG-HRP (1/6000 in PBST) was added (100 μL/well). After this step, the plates were processed as described in the general protocol.

Cross-Reactivity Determinations. Stock solutions of different phenolic compounds and anisoles were prepared in DMSO at a concentration of 1 mM. Standard curves were prepared for each analyte in DMSO (1000 μM to 6.4 nM) and diluted 200 times with A78 PBST−EtOH solution as indicated before. Each IC_{50} value was determined in the competitive experiments following the optimized protocol described above. The cross-reactivity values were calculated according to the equation: (IC_{50} 2,4,6-TCA/IC_{50} phenolic or anisole derivative) × 100.

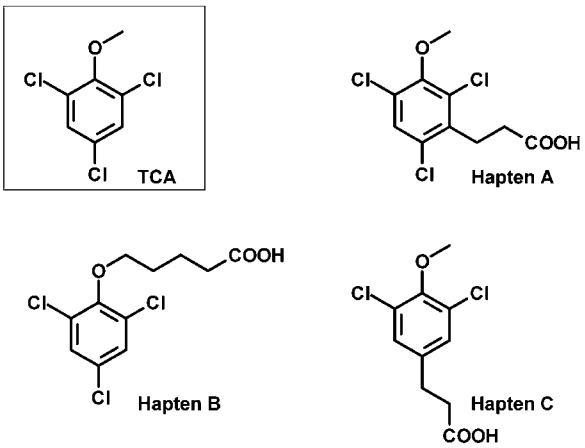

Figure 1. Chemical structures of the immunizing haptens **A**, **B**, and **C** and the target analyte, TCA.

Accuracy. *Blind Spiked Samples.* This parameter was assessed by preparing nine different blind spiked samples in PBS and measuring them with the optimized ELISA. Analyses were made in triplicate.

Matrix Effect Studies. On a first step, the pH values of the wines used in this study were adjusted to 8.5 with 5 N NaOH. Similarly, the conductivity was adjusted to 15 mS cm^{-1} using a 0.2 M NaCl aqueous solution. Following, the As78 was diluted in raw wine or wine serially diluted with PBST−EtOH. The standards used to calibrate the assay, which had been previously prepared in DMSO (1000 μM to 6.4 nM), were then diluted 200 times in these solutions before adding them to the plates. The curves were run according to the conditions described above for the optimized assay As78/**C9**-OVA, and the absorbances obtained were adjusted to the four parameter equation to compare their parallelism to the curve prepared in the absence of wine.

RESULTS AND DISCUSSION

In our previous paper, we designed and synthesized three immunizing haptens showing a distinct type of resemblance to the target analyte in order to study their ability to provide usable immunoassays. Thus, with respect to the analyte, hapten **A** preserved all of the functional groups, hapten **B** had exactly the same electronic behavior, and hapten **C** better preserved the geometry of TCA (see **Figure 1**). The antisera As74−76, As77−79, and As88−90 were obtained from **A**-KLH, **B**-KLH, and **C**-KLH, respectively. As result of these studies, we found that hapten **C** was the immunizing hapten rendering the highest amount of competitive immunoassays with acceptable parameters (A_{max} 0.5−1.2 units of absorbance, slope > 0.5, signal-to-noise ratio > 5, and IC_{50} values lower than 10 μg L^{-1}) on that screening step. We explained this particular result by the greatest exposure to the immune system of the region defined by the methoxy group and the two chlorine atoms at *ortho* positions. The spacer arm placed in *para*, the more distal part to this area, was responsible for this effect. In this paper, we present the evaluation of these assays considering several physicochemical features. With this purpose, the best antiserum/coating antigen combination (As76/**C14**-CONA, As78/**C9**-OVA, and As88/**C**-OVA) obtained from each immunizing hapten was selected for further studies.

Reproducibility was the first effect to consider. **Table 1** shows the variation of the parameters of the standard curves when the immunoassays were assayed on different days. The extraordinary variability of the detectability observed was remarkable, particularly for those assays reaching better limits of detection. Thus, the detectability (in terms of IC_{50}) of the immunoassay As88/**C**-OVA varied from 0.3 ng L^{-1} to 0.2 μg L^{-1} ($N = 3$ days; 0.04 ± 0.1 μg L^{-1}, the SD is 143% of the average value).

Table 1. Day to Day Reproducibility of the Immunoassays Selected for Each Immunogen[a]

immunogen	assay	day	A_{max}	A_{min}	slope	IC_{50} ($\mu g\ L^{-1}$)	R^2
A-KLH	As76/**C14**-CONA	1	1.129	0.076	–0.51	2.29	0.980
		2	1.384	0.109	–0.57	0.047	0.982
		3	0.872	0.004	–0.60	5.66	0.987
B-KLH	As78/**C9**-OVA	1	1.600	0.328	–0.80	9.73	0.980
		2	1.439	0.237	–0.087	7.50	0.996
		3	1.489	0.032	–1.07	6.30	0.980
C-KLH	As88/**C**-OVA	1	0.618	0.017	–0.92	0.19	0.991
		2	0.651	0.030	–0.44	0.022	0.930
		3	1.143	0.175	–0.86	3×10^{-4}	0.781

[a] The features shown in the table were extracted from the four parameter equation used to fit the standard curve prepared every day. The assays were performed using two well duplicates on different nonconsecutive days.

Table 2. Physicochemical Features[a] of TCA and Other Analytes for Which ELISAs Have Been Reported

compound	vapor pressure (mmHg)	Log P	water solubility ($mg\ L^{-1}$)	ELISA (ref)
benzene	94.8	2.13	1790	*20*
toluene	28.4	2.73	526	*20*
xylenes	7.99	3.16	106	*20*
2,4,6-TCA	$2.28\ 10^{-2}$	4.11	10.0	
2,4,6-TCP	$8.00\ 10^{-3}$	3.69	800	*21*
chlorpyrifos	$2.02\ 10^{-5}$	4.96	1.12	*25*
atrazine	$2.00\ 10^{-7}$	2.61	34.7	*23*

[a] Data extracted from Merck Index.

The most reproducible assay was As78/**C9**-CONA ($N = 3$ days; $7.84 \pm 1.74\ \mu g\ L^{-1}$, SD is 22%), although the detectability was much lower than the others (higher IC_{50}). This lack of reproducibility complicated the evaluation and application of these assays. Therefore, As76/**C14**-CONA was selected for further studies as a compromise between detectability and reproducibility ($N = 3$ days; $2.66 \pm 2.82\ \mu g\ L^{-1}$, SD is 106%) in order to investigate the reasons for this behavior.

Evaluation of the Immunoassay As76/C14-CONA (Immunogen A-KLH): Effect of the TCA Properties. Three major problems were continuously observed in this assay: the random appearance of the "Hook effect", the low slope values, and the insufficient day to day reproducibility (see **Table 1**). The Hook effect or prozone phenomenon consists of the unexpected paradoxical decrease of the absorbance at concentrations lower than a certain value of the calibration curve. The origin of it is uncertain, although in noncompetitive assays it has been speculated that it may be related to the antigen−solid phase interaction (*15*−*19*).

Because the lack of reproducibility seemed to be a common factor in all of the combinations assayed, especially those showing better detectability (see **Table 1**), we started to question if the particular features of TCA (volatility and lipophilicity) could be one of the reasons. Thus, a bench of substances showing different physicochemical features and to which ELISAs had been reported (benzene, xylenes, and toluene (*20*), 2,4,6-TCP (*21*, *22*), atrazine (*23*), and chlropyrifos (*24*, *25*)) were selected and their behavior was compared to that of TCA (see **Table 2**). As it is shown, TCA has a pressure of vapor of 2.28×10^{-2} mmHg, which is higher than that of other compounds for which robust immunochemical methods have been described (chloropyrifos, atrazine, and 2,4,6-TCP) but not as much as for benzene, toluene, or xylene. Beyer and co-workers (*20*) also associated the lack of reproducibility of the benzene, toluene, and xylene immunoassays to this particular aspect mentioning the possibility that losses of the analytes could occur while handling and performing the assay. Thus, trace determination of these volatile analytes seemed to be strongly influenced by this physicochemical feature. To prevent this problem, they performed the competitive step at 4 °C. However, following their experience, the IC_{50} of our assay dropped from about 3 to 130 $\mu g\ L^{-1}$. Using longer incubation periods to compensate for the effect of the temperature did not improve immunoassay performance.

Table 3. Effect of the Organic Solvents on the Features of the As76/**C14**-CONA Immunoassay[a]

solvent	day	A_{max}	A_{min}	slope	IC_{50} ($\mu g\ L^{-1}$)	R^2
PBST[b]		0.999 ± 0.256	0.055 ± 0.038	-0.59 ± 0.27	8.9 ± 9.35	0.930 ± 0.05
DMSO	1	1.000	0.034	–0.54	3.96	0.871
	2	1.180	0.001	–0.49	3.73	0.993
dioxane	1	0.654	0.036	–1.17	2.79	0.890
	2	0.664	0.088	–0.56	4.31	0.942
DMF	1	0.726	0.004	–0.89	17.1	0.967
ethanol	1	0.682	0.008	–2.54	45.3	0.908

[a] The standard curve was prepared in the organic solvents and then diluted 200 times with the antibody solution in buffer prior to their addition to the coated plate The parameters shown were extracted from the four parameter equation used to fit the standard curve. A fresh curve was prepared every day, and the curves were run using two well duplicates. [b] The parameters shown for the curve prepared in PBST correspond to the average of the five curves prepared in five consecutive days. For this case, the curve was analyzed using three well replicates.

Another potential drawback for the immunochemical determination of TCA was its lipophilicity. As can be observed in **Table 2**, TCA has a high log P value (4.11) if compared to other analytes (atrazine, 2.61; benzene, 2.13; etc.). In fact, for the analytes selected, it occupies the second place after chlorpyrifos (log P, 4.96). It is well-known that lipophilic substances tend to adsorb to surfaces. The use of plasticware, particularly if low concentrations have to be determined, should be avoided in those cases. Thus, Johnson-Longan and co-workers made a systematic study on the absorption of the insecticide chlordane (log P, 5.58) to labware showing that this nonpolar compound was adsorbed to polypropylene 87% and to glass-treated surfaces in 22% (*26*). Because plasticware is the usual type of material employed on immunochemical analysis (tubes, microplates, pipet tips, etc.), this fact was contemplated as a potential main source of error. Manclús and co-workers also avoided as much as possible the use of plasticware while working with the chlorpyrifos immunoassay (personal communication). Moreover, because of the low water solubility associated with lipophilic substances (see **Table 2**), they proposed the preparation of the chlorpyrifos standard curve in an organic solvent instead of the usual assay buffer. They attributed their observed day to day immunoassay variability to the errors introduced while preparing the standard curve by performing serial dilutions in water (*24*, *25*). Therefore, the effect of preparing the standard curve of TCA in different water miscible solvents (DMSO, dimethylformamide (DMF), ethanol, and dioxane) was investigated. The standards were prepared in organic solvents and further diluted with buffer to a 0.5% content prior the ELISA analysis. The results from this study are shown in **Table 3**. It can be observed that the use of ethanol and DMF increased the value of the IC_{50} (lower detectability). In contrast, both DMSO and dioxane produced a positive effect on the ELISA features and also on its reproducibility. Plasticware was

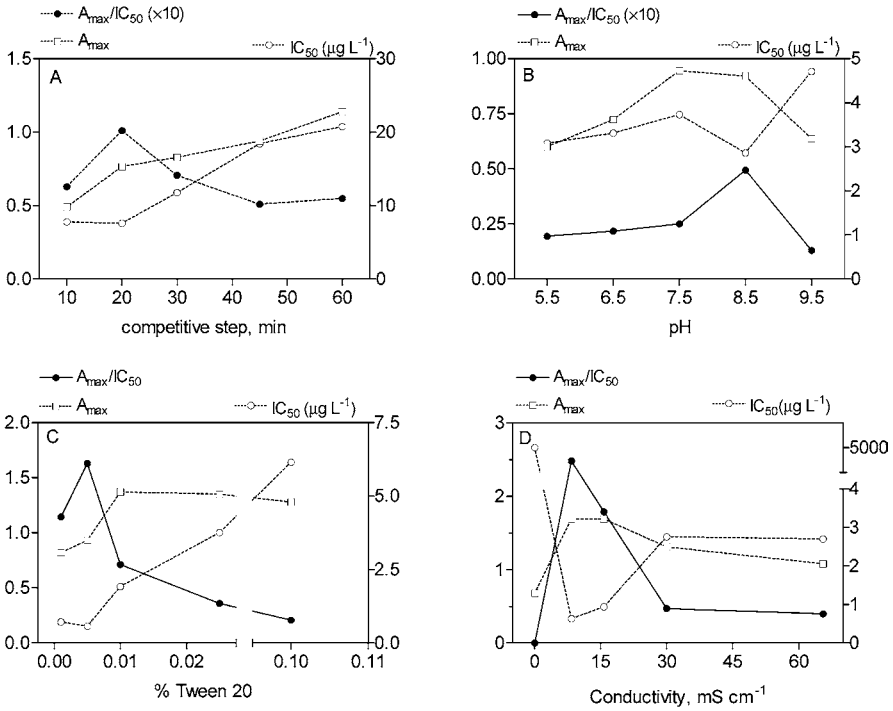

Figure 2. Graphs showing the influence of several parameters on the performance of immunoassay As78/**C9**-OVA. (**A**) Effect of the length of the competitive step; (**B**) effect of the pH; (**C**) effect of the detergent concentration; and (**D**) effect of the ionic strength. The data presented are extracted from the four parameter equation used to fit the standard curve. Standard curves were prepared using two well replicates.

always avoided, except for the pipet tips, since their exclusion from the normal microtiter plate ELISAs protocols would be extremely difficult to practice.

In the same line of troubleshooting the TCA immunoassay, we found that Jung and co-workers had to prepare each day a fresh standard curve for the fungicide fenpropimorph to maintain the immunoassay reproducibility (*27*). Fenpropimorph is a very high lipophilic substance with a log P of 4.88. Similarly, Hill et al. described that the number of freeze−thaw cycles significantly affected the concentration of a stock solution of chlorpyrifos (*28*). Keeping the TCA standard curve or the stock solutions prepared in DMSO in the refrigerator could be another reason for the variability observed in our assay (because of the low melting point of this solvent, it remains frozen when stored in the refrigerator at 4 °C). Thus, the evolution of the IC_{50} values recorded after running the assay during six consecutive days was as follows: 1.39, 2.11, 8.24, 13.5, 15.7, and 22.4. Freeze and thaw cycles of the TCA stock solution occurred each time that we performed an assay, since this solution was stored in the refrigerator. The same stock solution could not be used more than two times when stored at 4 °C. In contrast, despite the TCA volatility, we found that the DMSO solutions were quite stable if stored in closed vials but at room temperature.

Despite all of the cautions taken after all of these studies, the reproducibility accomplished, although better than before, was not completely satisfactory ($N = 6$ days; 1.06 ± 074 μg L^{-1}, the SD is 70% of the average value). Moreover, the assay slope was still too low for a quantitative immunoassay ($N = 6$ days; -0.48 ± 0.10).

At this point, we were also concerned about the contamination of the laboratory atmosphere with TCA since some synthetic steps, involving working with high TCA amounts, and all of the TCA stock solutions had been prepared in the same laboratory. Additionally, experts on TCA organoleptic analysis from the cork industry that had been collaborating on this project noticed a musty aroma in the laboratory. This fact could also interfere with the quantitation of TCA at low concentration levels and could have contributed to the low reproducibility of the assays developed using antibodies raised against **C**-KLH. For this reason, we decided to move forward by applying all of this knowledge to the As78/**C9**-OVA assay, which, although its detectability was lower, showed a much higher reproducibility day to day (see **Table 1**). This opened the possibility for a more reliable evaluation of other physicochemical parameters that would help to improve immunoassay performance.

Evaluation of the Immunoassay As78/C9-OVA (Immunogen B-KLH). Paying attention to all of the above factors related to the TCA features, we started by evaluating the effect of the length of the competitive step. As observed in other examples described in the literature, the time given to the immunoreagents to interact resulted in a direct effect on the curve parameters (*23*, *29*) (see **Figure 2A**). Incubation periods longer than 30 min produced a decrease of the immunoassay detectability (in terms of IC_{50}) and an increase on its maximal absorbance. An incubation period of 20 min was chosen as a compromise between detectability and maximal absorbance of the assay. Contrary to other immunoassays described for triazines (*23*, *29*, *30*), when the analyte was preincubated with the antibody, no improvement in the immunoassay detectability was observed (IC_{50} values recorded as follows: 24 h at 0 °C, 11.21 μg L^{-1}; 2 h at RT, 13.66 μg L^{-1}; 1 h at RT, 8.9 μg L^{-1}). The high background frequently observed in this assay (see **Table 1**) probably due to nonspecific interactions was minimized by the use of 1% BSA, 1% OVA, 1% skimmed milk, and 1% PVP−0.05% Tween as blocking agents. In all cases, we succeeded reducing the minimum absorbance of the assay to values below 0.04 units of absorbance. However, a significant decrease of the maximal absorbance of the immunoassay was also observed, except for the case of the PVP−Tween. Regarding effect of the pH, the assay was quite stable in the range between 5.5 and 9.5, although the best performance occurred at pH values between 7.5 and 8.5. Outside this interval, the

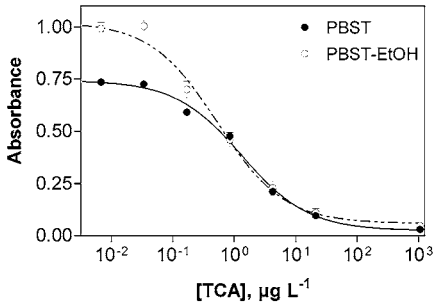

Figure 3. Calibration curve of the optimized 2,4,6-TCA immunoassay As78/**C9**-OVA run in PBST and in PBST–EtOH. The data presented correspond to the average and standard deviation of eight assays, run on different days. The curves were prepared using two well duplicates. See **Table 4** for the features of the immunoassay run in both media.

maximum absorbance decreased (see **Figure 2B**). We also found that the TCA immunoassay As78/**C9**-OVA was strongly influenced by the concentration of Tween 20 used as a nonionic detergent in the assay. Thus, concentrations below 0.01% significantly improved the immunoassay detectability. However, a decrease of the slope value was also advertised if the percentages were lower than 0.005%, and in the absence of detergent, the IC_{50} value drastically increased again while increasing the coefficient of variation between the different replicates (see **Figure 2C**). These results are in accordance with those obtained by Manclús et al. when they developed the chlorpyrifos immunoassay (*25*). In this case, not only the detectability improved when decreasing the Tween 20% but also an increase in the absorbance was observed, allowing higher dilutions of the immunoreagents. To explain this strong influence of the nonionic detergent on the immunoassay properties, the establishment of nonspecific hydrophobic interactions between the nonpolar analytes and the detergent molecules has been postulated, which could compete with the specific interaction analyte−antibody (*31*, *32*). This would explain the enhancement of the antibody−analyte interaction when diminishing the concentration of Tween 20. Finally, different from what has been reported on other immunoassays for phenolic compounds (*21*, *33*, *34*) or for triazines (*35*), this assay was not significantly affected by the ionic strength within the range of 9−65 mS cm^{-1} (5−50 mM in the terms of PBS). However, in the absence of salts (e.g., MilliQ water, 6 μS cm^{-1}), the assay was completely inhibited (see **Figure 2D**). The best detectability values were accomplished between 9 and 16 mS cm^{-1}.

As a result of this evaluation, we were able to improve considerably the features of the immunoassay As78/**C9**-OVA. We must remark that we took all of the necessary precautions regarding handling of TCA according to our experience evaluating the immunoassay As76/**C14**-CONA (see above). This could have helped the reliability of the results obtained in this second assay. **Figure 3** shows the curve corresponding to the optimized immunoassay As78/**C9**-OVA, and **Table 4** summarizes the parameters defining the calibration graph of the ELISA. The values shown correspond to the average and standard deviation of the assay performed on different days ($N = 6$) demonstrating the higher reproducibility (SD is 14%) of this assay under the conditions established after the evaluation was made. The limit of detection (LOD) accomplished was 77 ng L^{-1}, which is near the sensorial threshold estimated for the presence of TCA in red wines (50 ng L^{-1}). At this point, we also evaluated the effect of ethanol in the assay since wine samples are one of the main targets of this assay. The result of this study showed that a 7% ethanol produced a significant improvement in the immunoassay

Table 4. Features of the Optimized Immunoassay As78/**C9**-OVA[a]

	PBST[b]	PBST (7% EtOH)[c]
A_{min}	0.024 ± 0.018	0.058 ± 0.029
A_{max}	0.743 ± 0.057	1.027 ± 0.086
slope	–0.80 ± 0.11	–0.80 ± 0.20
IC_{50} (μg L^{-1})	1.32 ± 0.19	0.53 ± 0.14
LOD (μg L^{-1})	0.077 ± 0.038	0.044 ± 0.028
dynamic range (μg L^{-1})	6.83 ± 1.10 to 0.22 ± 0.06	3.33 ± 0.47 to 0.10 ± 0.04
R^2	0.995 ± 0.005	0.991 ± 0.006

[a] The parameters are extracted from the four parameter equation used to fit the standard curve. Each curve was built using two well replicates. [b] The data presented correspond to the average of six calibration curves run on six different days. [c] The data presented correspond to eight calibration curves run on eight different days.

Table 5. Interference Caused by Structurally Related Chemicals on the As78/**C9**-OVA Immunoassay, Expressed by Their IC_{50} Values and the Percentage of Cross-Reactivity (CR%)[a]

no. halogens[b]	compound	log P	IC_{50} (nM)	% CR
3	2,4,6-TCA	4.11	6.29	100
1	3-CP	2.40	>5000	<0.1
	4-CP	2.43	>5000	<0.1
2	2,4-DCP	2.99	>5000	<0.1
3	2,4,5-TCP	3.71	>5000	<0.1
	2,4,6-TCP	3.58	>5000	<0.1
4	2,3,4,6-TtCP	4.17	>5000	<0.1
5	PCP	4.78	>5000	<0.1
2	2,3-DCA	3.39	>5000	<0.1
	3,5-DCA	3.80	>5000	<0.1
	2,6-DCA	3.26	>5000	<0.1
4	2,3,4,5-TtCA	4.61	>5000	<0.1
3	2,4,6-TBA	4.14	3.15	200

[a] Cross-reactivity is expressed as a percent of the IC_{50} of the 2,4,6-TCA divided by the IC_{50} of the halogenated compound. Log P values have been included for discussion. The cross-reactivity studies were performed with the ELISA in the absence of ethanol in the competition buffer. [b] B, bromo; C, chloro; CP, chlorophenol; DCP, dichlorophenol; TtCP, tetrachlorophenol; PCP, pentachlorophenol; DCA, dichloroanisole; TtCA, tetrachloroanisole.

features reaching a LOD of 44 ng L^{-1} (see **Figure 3** and **Table 4**). However, percentages greater than 10% rendered higher IC_{50} values while 35% of ethanol inhibited the assay.

Immunoassay Specificity. Although 2,4,6-TCA has been identified as the main agent responsible for the cork taint problem (*36*), other structurally related chemicals have been reported to contribute to this musty odor (*37*, *38*). Therefore, it was very important to evaluate the potential interference of other structurally related compounds also potentially present in wine. Thus, other chloroanisoles such as 2,3,4,6-tetrachloroanisol (2,3,4,6-TeCA) may also be present due to their common origin on the chlorophenol formulations used as insecticides and wood preservatives. After 12 compounds with chemical structures related to TCA (seven chlorophenols and five chloroanisoles) were assayed, it was concluded that the interference produced by the coexistence of these compounds in the matrix was negligible (see **Table 5**). Any of the chlorophenols assayed were recognized in this assay. Thus, 2,4,6-TCP, possessing the same distribution for the chlorine atoms, did not cross-react at concentrations as high as 5000 nM. These results demonstrate that the methoxy group is essential for antibody recognition. Only 2,4,6-tribromoanisole (2,4,6-TBA) was highly recognized in this assay (%CR = 200). An increasing interest in the detection of brominated anisoles has arisen in the last years on certain samples since its sensory threshold has been shown to

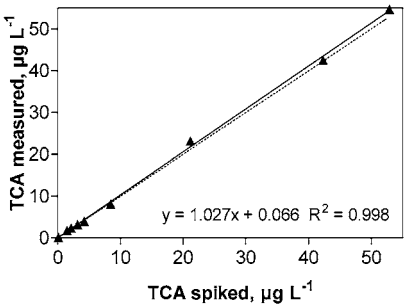

Figure 4. Graph showing the correlation between the spiked concentrations and the values measured by ELISA. The data shown correspond to the average three well replicates. The dotted line corresponds to a perfect correlation (slope = 1).

be even lower than 2,4,6-TCA, also associated with similar and undesirable musty and moldy aromas (*4*, *38*). However, it is very much unlikely that this compound could be found in wine and cork extract matrixes since the origin of these brominated organic compounds is recent and it has been mostly related to their use as flame retardants in plastics, treated woods, or textiles. The greater recognition of brominated analytes analogues has already been reported in our group for the 2,4,6-TCP indirect and direct ELISAs (*21*, *22*). The role of the hydrophobic interactions in the stabilization of the immunocomplex when considering nonpolar analytes has been regarded as a reasonable explanation for this behavior (*22*, *39*). This fact agrees with the 2,4,6-TBA and 2,4,6-TCA log P values.

Immunoassay Accuracy. Nine blind samples were prepared in water containing 7% ethanol and analyzed using the optimized As78/**C9**-OVA ELISA after buffering the samples to adjust for conductivity and pH values. The correlation between the spiked and the measured values was very good as can be observed in **Figure 4** with a regression coefficient of $R^2 = 0.998$ and a slope value of 1.027.

Matrix Effect Studies. 2,4,6-TCA has been found in river (*40*) and drinking waters (*41*−*43*). Although its contribution to the decrease of the water quality has been well-established, nowadays, the magnitude of the problem is not comparable with the economic losses originated in wine and cork samples in the wine industry (*44*). For this reason, one of our ultimate objectives was measuring 2,4,6-TCA in wine samples. To assess the effect of these type of matrixes on the As78/**C9**-OVA immunoassay, we selected representative white wine and red wine samples. In the first instance, the pH, conductivity, and ethanol percentage of both wines were adjusted to place the samples within the working conditions of the immunoassay. Following, standard curves were prepared in raw wine and PBST−EtOH diluted wine to compare their parallelism to the curve prepared in PBST−EtOH. As shown in **Figure 5**, a significant decrease of the maximum absorbance was observed when the assay was run in the presence of wine. By using the same matrix as the immunoassay solvent, red wine and white wine samples had to be diluted at least 25 and five times, respectively, to get the assay to work. Under these conditions, the LOD values would be set at about 200 ng L^{-1} and 1 μg L^{-1} for white and red wines, respectively. However, because it is questionable that all wine varieties will produce the same effect on the immunoassay, white and red wines would had to be diluted over 50 and 100 times, respectively, to ensure reliability. By applying this dilution factor, the standard curve could be prepared in PBST−EtOH although the detectability would obviously be compromised. The LOD values would then be around 2 and 4 μg L^{-1} for white and red wines, respectively,

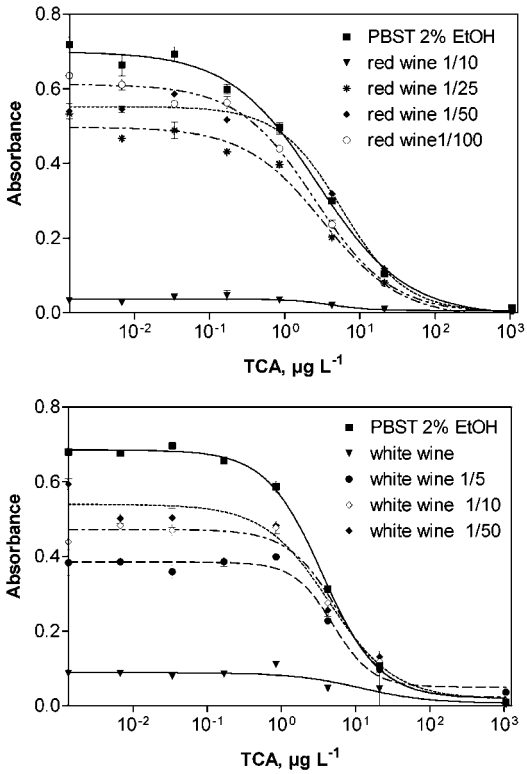

Figure 5. Calibration graphs showing the interference produced by the white and red wine samples tested in the ELISA As78/**C9**-OVA.

which are clearly insufficient for direct analysis, considering the low sensory levels for TCA in these matrixes. These results raise the need of developing suitable sample treatment methods prior to the immunochemical analysis. By coupling an efficient cleanup method with the immunoassay presented here, simultaneous measurement of many samples would then be possible, increasing the efficiency of the monitoring methods.

CONCLUSIONS

Several ELISAs have been developed for TCA determination. Those assays showing higher detectability were associated to a lack of reproducibility. Knowledge of the particular physicochemical properties of this analyte has been crucial to establish a method that is reproducible and reliable. Thus, contact with plastic surfaces should be avoided and the standards have to be prepared in organic media. Evaluation of the immunoassay As78/**C9**-OVA has demonstrated that the assay is operative at pH values ranging from 6.5 to 9.5, although the best features are accomplished within the pH range of 7.5−8.5. On the other hand, as occurred with other lipophilic substances, the percentage of Tween 20 should be kept to a minimum to ensure solubility and low coefficients of variation, without affecting antibody recognition. The assay is very specific to TCA since does not recognize other potential chlorinated contaminants in wine. From all of the substances studied, only 2,4,6-tribromoanisol interferes in this assay, although this compound is very much unlikely to be found in wine. Unfortunately, wine matrixes produce important interferences in the immunoassay preventing us from directly using this method to measure real samples without any previous treatment. In light of these results, cleanup procedures compatible with the immunoassay method have to be developed to eliminate this undesirable effect. Because of the high lipophilic character of TCA and its tendency to adsorb to solid surfaces, the use of simple methods frequently used in

wine analysis, such as tannin precipitation, does not look promising. Similarly, sample treatment procedures involving steps where the organic solvent would have to be evaporated may lead to low recoveries due to its high volatility. Using trapping solvents such as propylene glycol or DMSO may help to reduce this effect. Another strategy is to use affinity-based extraction methods. An immunoaffinity cleanup procedure for the analysis of ochratoxin A in wines has recently been described (*45*). Immunoaffinity chromatography has been shown to combine the advantages of other solid phase extraction procedures while providing the specificity inherent to the antibody−antigen interaction (*46*−*49*). Additionally, it provides aqueous extracts that are compatible with the immunoassay conditions. Therefore, this technique is regarded as a challenging strategy to overcome the interferences caused by the wine matrix in this immunoassay. Finally, we still consider the possibility to accomplish a greater reproducibility for the assays developed with the antisera raised against **C**-KLH. The high detectability of these immunoassays would allow direct analysis of wine samples just after application of an appropriate dilution factor in order to avoid interferences from the sample matrix.

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